S. Thomas 7/19

Colorado State University

College of Veterinary Medicine
and Biomedical Sciences
Department of Microbiology
Fort Collins, Colorado 80523
Telephone: (303) 491-6136
FAX: (303) 491-1815

June 27, 1995

Dr. Diane Spindler
NREL
1617 Cole Blvd.
Golden, CO 80401-3393

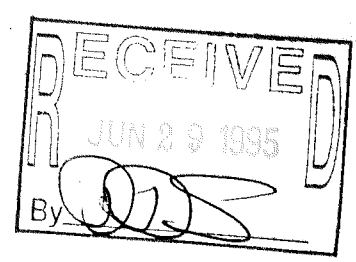
Re: Subcontract No. XAC-3-11185-01 "Identification of Leading Cellulase Producing Microorganisms" 07/27/93-07/26/95

Dear Dr. Spindler:

Please find enclosed the Final Report on this project. The report encompasses work completed in a two year period, and incorporates answers to review comments made on Annual Report 4/10/95. If you have further questions, address them to me in Hungary between July 10 and November 14, 1995.

My address:

Dr. Robert P. Tengerdy
c/o Dr. G. Szakacs
Techn. Univ. Budapest
Dept. Agr. Chem. Technol.
H-1521 Budapest
Gellert ter 4
Hungary
Phone: 36-1-463-1475/4030
Fax: 36-1-463-2598
Email: SZAKACS@ch.bme.hu



Sincerely,

Robert P. Tengerdy
Professor Emeritus

RPT:lam
C. Rasey
S. Thomas

FINAL REPORT

CONTRACTING PARTY: NATIONAL RENEWABLE ENERGY LABORATORY

SUBCONTRACT NO.: XAC-3-11185-01

TITLE: IDENTIFICATION OF LEADING CELLULASE PRODUCING MICROORGANISMS

SUBCONTRACTOR: Robert P. Tengerdy
Professor Emeritus
Department of Microbiology
Colorado State University
Fort Collins, CO 80523-1677

TECHNICAL MONITOR AT NREL: Dianne Koepping/Diane Spindler

PERIOD OF PERFORMANCE: 07/27/93 - 07/26/95

DATE SUBMITTED:

SUBMITTED BY:

SUMMARY

Five lignocellulolytic fungi were isolated and selected from an extensive screening program including over 400 fungi. The selected fungi produced higher cellulase activity and much higher beta glucosidase activity than the wild parent strain of the high cellulase producing mutant, *Trichoderma reesei* Rut-C30. Two of the isolated wild strains produced more cellulase and beta glucosidase than Rut-C30 in a 4 day solid substrate fermentation. This research was conducted in collaboration with Dr. G. Szakacs at the Technical University of Budapest, Hungary, whose culture collection was used for this study.

INTRODUCTION

The purpose of this research was to isolate and identify new wild strains of lignocellulolytic fungi with high potential for cellulase production and lignocellulose degradation. The selection criteria were: 1. cellulase productivity higher than that of the parent strain of *Trichoderma reesei* Rut C30, the selected reference strain; 2. a ratio of cellulase/beta glucosidase favorable for lignocellulose degradation; 3. an enzyme composition that is amenable for strain improvement by genetic manipulation; 4. substrate specificity, in this case, suitability for growth and enzyme production on pretreated poplar wood. In the course of the study a fifth selection criterium was added, growth and enzyme production on natural solid substrates, as a means for economical in situ enzyme production (1). The screening involved over 400 fungi that were collected worldwide previously or during the course of this study, and deposited in the culture collection of Dr. G. Szakacs at the Technical University of Budapest.

METHODS

Substrates for Submerged Fermentation

Two carbon sources were used for screening and later testing both in shake flask and stirred tank fermentations: amorphous cellulose Solka Floc 10 IND (James River Co., Berlin, NH)at 10 g/l concentration (Medium A), and pretreated poplar wood (PPW) (supplied by the National Renewable Energy Laboratory (NREL), Golden, CO, with 77% cellulose content) used at 13 g/l concentration (dry weight (DW) basis) (Medium B). The composition of Medium A and B is given in Table 1.

Table 1. Composition of media used for cellulase production.

Ingredients	Concentration (g/l) Medium A	Concentration (g/l) Medium B
Solka Floc 10 IND	10	-
PPW*	-	13
Corn steep liquor*	1.0	1.0
KH_2PO_4	2.0	2.0
$(NH_4)_2$ HPO_4	1.5	1.5
Urea	0.3	0.3
$MgSO_4.7H_2O$	0.3	0.3
NaCl	0.5	0.5
$CaCl_2$	0.3	0.3
Tween 80	1.0	1.0
antifoam**	1.0	1.0
$MnSO_4$	$1.6x10^{-3}$	$1.6x10^{-3}$
$ZnSO_4.7H_2O$	$3.4x10^{-3}$	$3.4x10^{-3}$
$CoCl_2.6H_2O$	$2.0x10^{-3}$	$2.0x10^{-3}$
$FeSO_4.7H_2O$	$5.0x10^{-3}$	$5.0x10^{-3}$

*dry weight basis

** Struktol, Richter G. AG., polyoleate type

Substrates for Solid Substrate Fermentation

Extracted sweet sorghum silage was obtained from the bioprocessing of sweet sorghum by enzyme assisted ensiling (ENLAC), followed by countercurrent diffusion for sugar extraction (10). This substrate was selected, because it is used for in situ enzyme production in the bioprocessing of sweet sorghum by ENLAC (1). The extracted and dried silage contains about 25% available carbon in the form of cellulose and hemicellulose, and about 0.6% nitrogen in the form of protein. Wheat straw, supplemented with 10% wheat bran contains about 30% available carbon and 0.9% nitrogen. The dried substrates were milled to about 0.5 cm size and supplemented with the following salt solution: NH_4NO_3:5, KH_2PO_4:5, $MgSO_4.7H_2O$:1, NaCl:1g/l; $MnSO_4$:1.6, $ZnSO_4.7H_2O$:3.5, $CoCl_2.6H_2O$:2.0, $FeSO_4.7H_2O$:5.0mg/l. For 1g dry sustrate 3 ml salt solution was added to give a moisture content of 75% and an approximate C:N ratio of 25 for sweet sorghum and 18 for wheat straw/bran. The initial pH before sterilization was 4.8.

Submerged Fermentation

For shake flask fermentation, 150 ml medium was distributed into 750 ml Erlenmeyer flasks, and sterilized at 121°C for 15 min. The final pH was adjusted to 4.8. The flasks were inoculated with a spore suspension of the test fungi to give a spore concentration of 10^5 Colony Forming Unit (CFU) per ml final medium. The flasks were incubated on a rotary shaker at 28°C and 220 rpm for 4-5 days.

The most promising strains were tested by submerged fermentation in 15 l stirred tank (ST) bioreactors (New Brunswick, NJ) with 7-10 l useful volume, using the same media as in shake flasks. The media were inoculated with 5% (v/v) of a 48h shake flask culture of the respective fungus and the ST reactor was operated for 4 days with the following parameters: t=28°, pH=4.8 (maintained manually or with pH controller in later experiments); aeration=0.5

v/v, min; agitation=300 rpm.

Solid Substrate Fermentation (SSF)

SSF was performed in 250 ml double plastic cups, the inner cup having perforations for air access, the outer providing protection from contamination. The prepared substrate was loosely packed into the inner cup (80 g/cup for sweet sorghum, 70 g/cup for wheat straw/bran), sterilized at 121°C for 20 min, then inoculated with the spore suspension of the test fungi to a final concentration of 10^7 spores/g DW. The spore suspension was prepared by washing spores from the surface of 2 weeks old sporulating agar plate cultures of the respective fungi with 0.1% Tween 80 containing water, and concentrated to $1.0x10^8$ spores/ml. 0.1 ml of this suspension was used per g DW substrate to give a final spore count of 10^7 spores/g DW. The inoculated cups were incubated at 30°C in a 99% relative humidity chamber for 10 days.

Larger scale SSF with the best fungi was performed in aluminum trays (780 x 510 x 80mm), 500g DW prepared substrate (2000g wet substrate at 75% moisture content) per tray in a layer thickness of about 5 cm. SSF was performed as above for 6 days at 30°C in a 99% relative humidity chamber. During incubation the trays were covered with filter paper, and raked daily for better distribution of mycelial growth and for ventilation.

Analytical Procedures

Fungal growth was followed by measuring the insoluble N content (protein content) of the fermentum by a modified Kjeldahl procedure (2). The N value was multiplied by 6.25 to get the protein content and by 2.7 to get the estimated biomass content, assuming that 37% of the fungal mycelium is protein (average value observed for 2-3 days growth of *T. reesei* Rut-C30). Soluble protein, indicative of secretion capability of a fungus, was measured by the bicinchoninic acid method (3). Enzyme activities were determined from the culture supernatant of submerged fermentations or the culture extract of SSF samples: 5g DW fermented substrate

was extracted with 95 ml water, containing 0.1% Tween 80, by shaking for 60 min. From the centrifuged supernatant or extract, filter paper activity (FPA) and beta glucosidase activity (BG) were determined by the standard methods used at NREL. These methods are identical with those reported in the literature for FPA (4) and BG (5). Beta 1,4 endoglucanase activity (EG) was determined by the method of Bailey and Nevalainen (6). Xylanase activity (Xyl) was determined according to Bailey et al. (7). Each variable was tested in four reps and each assay was done in duplicate. The means of these tests are shown in the results.

RESULTS AND DISCUSSION

The origin and brief characterization of the five selected strains is given below.

1. *Trichoderma hamatum* TUB F-105 (ATCC 62392)
   The strain was isolated from decaying reed, Hungary by G. Szakacs.
   Identification: *Trichoderma hamatum* (by L. Vajna, Budapest)
   The strain has been deposited at the American Type Culture Collection (ATCC) in 1986.

2. Unidentified fungus TUB F-426
   Isolation source: soil, Queensland, Australia
   Isolated by G. Szakacs
   Identification is in progress.
   A slow growing strain on PDA and natural substrates.

3. Trichoderma sp. TUB F-482
   The strain was isolated from wet forest soil, Florida by G. Szakacs.
   The culture has been identified as Trichoderma; species identification is in progress.
   Growth and sporulation are adequate both on laboratory and natural substrates.

4. Trichoderma sp. TUB F-486
   The strain was isolated from decaying wood, near Lake Placid, N.Y., by G. Szakacs.
   The culture has been identified as Trichoderma; species identification is in progress.
   A fast grower, sporulates well.

5. Gliocladium sp. TUB F-498
   The culture has been isolated from soil in Germany.
   The culture grows rapidly on laboratory media and natural substrates and sporulates well in 5 days. The culture has been identified as Gliocladium: species identification is in progress.

The reference strains were *Trichoderma reesei* QM6a and the mutant developed from it, Rut-C30. Four of the selected strains, F-105, F-482, F-486 and F-498, grew faster than QM6a and Rut-C30.

The results of shake flask fermentations with the five selected strains is shown in Table 2. All selected strains had higher overall cellulase activity than QM6a, the wild parent strain of Rut-C30 (8) on Solka Floc but not on PPW. All selected strains had higher beta glucosidase activity than Rut-C30. Two strains, TUB F-498 and TUB F-426, also had higher xylanase and 1,4 beta-endo-glucanase activity than Rut-C30 (data not shown). These data indicate that these selected wild strains have at least equal or greater potential for genetic improvement than QM6a had in a previous effort (8). Of all these wild strains, the *Gliocladium* sp. TUB F-498 seems to be the best adapted for enzyme production on PPW.

Table 2. Comparison of enzyme activities of selected fungi in shake flask fermentation

Strain	FPA Medium A	FPA Medium B	BG Medium A	BG Medium B
Trichoderma reesei Rut-C30	1.4±0.2	1.3±0.2	1.6±0.4	1.4±0.6
Trichoderma reesei QM6a	0.6±0.2	0.3±0.2	0.6±0.2	0.7±0.3
Trichoderma sp. TUB F-482	0.7±0.3	0.5±0.2	1.9±0.3	2.5±0.5
Trichoderma sp. TUB F-486	1.1±0.2	0.3±0.2	2.0±0.3	1.1±0.3
Trichoderma hamatum TUB F-105	1.2±0.2	0.3±0.1	2.8±0.3	1.8±0.2
Gliocladium sp. TUB F-498	1.1±0.2	0.7±0.3	2.2±0.4	2.4±0.2
Unidentified sp. TUB F-426	1.1±0.2	0.4	4.8±0.4	3.0±0.4

FPA: Filter paper activity IU/ml; BG: Beta glucosidase activity IU/ml.
Medium A contains Solka Floc 10IND, Medium B pretreated poplar wood as carbon sources; n=4-10. Fermentation time: 4 days.

Table 3. Comparison of biomass, enzyme production and enzyme secretion by selected fungi in stirred tank fermentation.

Strain	X		P		FPA		BG	
	Medium A	Medium B	Medium A	Medium B	Medium A	Medium B	Medium A	Medium B
Trichoderma reesei Rut-C30	3.1±0.4	2.7±0.5	2.1±0.2	1.6±0.7	1.4±0.2	1.3±0.2	1.2±0.2	1.2±0.4
Trichoderma sp. TUB F-482	3.4±0.3	3.4±0.6	1.5±0.2	1.1±0.3	0.6±0.3	0.6±0.3	1.5±0.3	2.7±0.6
Trichoderma sp. TUB F-486	2.2±0.5	2.1±0.4	-	1.2±0.3	0.5±0.3	0.6±0.3	0.6±0.5	1.9±0.7
Trichoderma hamatum TUB F-105	2.3±0.4	-	1.5±0.2	1.1±0.3	0.3	-	-	-
Gliocladium sp. TUB F-498	3.8±0.6	3.2±0.5	1.9±0.3	1.3±0.4	0.7±0.2	1.0±0.2	2.1±0.7	3.2±0.5
Unidentified sp. TUB F-426	2.4±0.2	2.5±0.7	2.0±0.4	0.9±0.5	0.9±0.6	0.6±0.2	4.8±0.9	3.1±0.6

FPA: filter paper activity IU/ml; BG: beta glucosidase activity IU/ml; X: biomass mg/ml; P: secreted protein mg/ml.
Medium A contains Solka Floc 10IND and Medium B pretreated poplar wood as carbon sources. n=4-12. Fermentation time: 4 days.

Table 4. Specific enzyme activities of selected fungi related to biomass and secreted protein production

Strain	FPA/X		FPA/P		BG/X		BG/P	
	Medium A	Medium B	Medium A	Medium B	Medium A	Medium B	Medium A	Medium B
Trichoderma reesei Rut-C30	0.4	0.5	0.7	0.8	0.4	0.4	0.6	0.7
Trichoderma sp. TUB F-482	0.2	0.2	0.4	0.5	0.4	0.8	1.0	2.4
Trichoderma sp. TUB F-486	0.2	0.3	-	0.5	0.3	0.9	-	1.6
Trichoderma hamatum TUB F-105	0.1	-	0.2	-	-	-	-	-
Gliocladium sp. TUB F-498	0.2	0.3	0.4	0.8	0.5	1.0	1.1	2.5
Unidentified sp. TUB F-426	0.4	0.2	0.4	0.7	2.0	1.2	2.4	3.4

The data of Table 3 were used for calculating the values. FPA: filter paper activity IU/ml; BG: beta glucosidase activity IU/ml; X: biomass mg/ml; P: secreted protein mg/ml. The ratios, FPA/X and BG/X are the specific enzyme activities per mg biomass produced; FPA/P and BG/P are the specific enzyme activities per mg secreted protein.

The performance of the five selected strains in ST fermentation is shown in Table 3. The enzyme activities were similar in shake flasks and ST reactors. The growth of the fungi was moderate on both substrates, slightly less on PPW than on Solka Floc. The fastest growth was observed with TUB F-498. The protein secretion capability of TUB F-498 and TUB F-426 was near equal with Rut-C30. All fungi secreted less protein on PPW than on Solka Floc. The specific enzyme activities related to biomass and secreted protein production are shown in Table 4. Rut-C30 was more efficient in cellulase production per unit biomass or unit secreted protein, but the wild strains, especially TUB F-498 and TUB F-426 were more efficient in beta glucosidase production. The time course of the fermentation with TUB F-498 is shown in Figure 1. Enzyme production and protein secretion coincided with fungal growth and reached maximum in about 4-5 days.

Table 5. Comparison of fungi for enzyme production in solid substrate fermentation

Strain	DW %	FPA	BG	Xyl	EG
Rut C30	24.8	3.2±0.3	0.3±0.1	480±18	550±25
TUB F-105	25.3	3.4±0.3	2.1±0.3	1300±35	580±30
TUB F-482	25.0	3.0±0.3	2.0±0.2	1000±42	500±42
TUB F-486	25.8	3.1±0.3	1.1±0.2	1050±53	520±28
TUB F-498	25.1	4.1±0.4	5.4±0.4	1470±53	710±40

Substrate: extracted sweet sorghum silage. Conditions: packing density 80 g/250 ml in perforated plastic cups; inoculum 10^7 spores/g substrate, moisture content 75%, temp 30°C. DW = dry weight; FPA = filter paper activity; Xyl = xylanase; BG = beta glucosidase; EG = endoglucanase; all activities in IU/g DW. Data were taken at 4 days; n=6; the means are shown with standard deviation.

The comparison of selected fungi for enzyme production on extracted sweet sorghum silage by SSF is shown in Table 5. In a relatively short 4 days fermentation TUB F-498

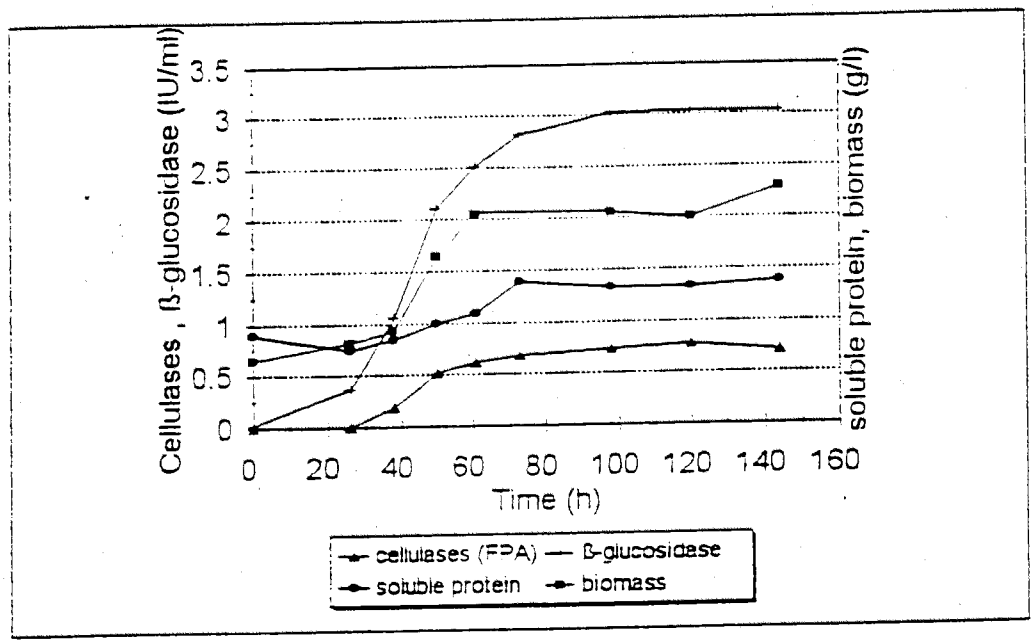
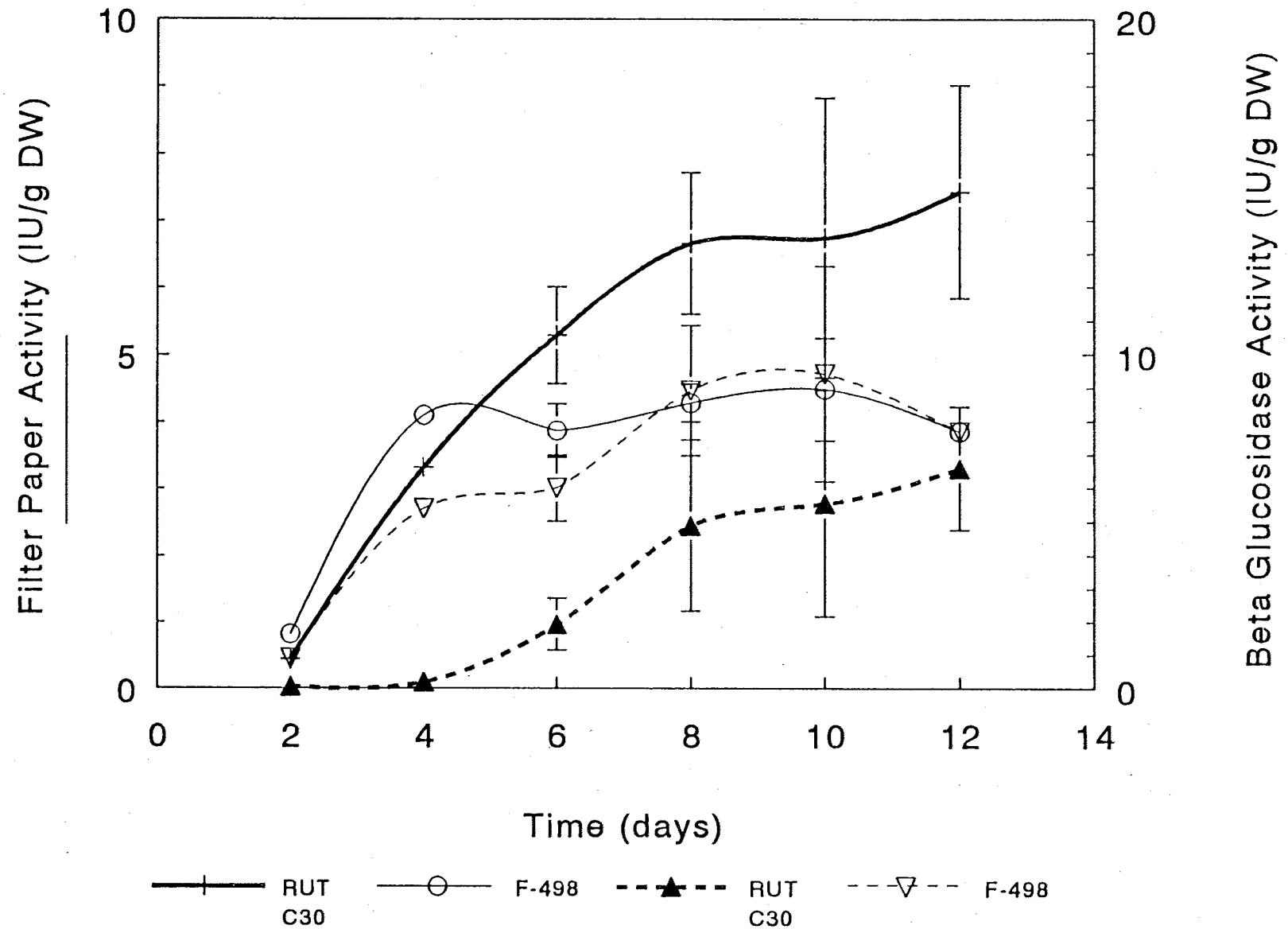


Figure 1. Biomass, protein secretion and enzyme activities of *Gliocladium* TUB F-498 in ST fermentation on pretreated poplar wood. V=7.0L; Air=0.5 v/v.min; pH=4.8; t=28°C. Inoculum: 5% v/v 48h mycelial culture.

Figure 2. Time course of enzyme production by *T. reesei* Rut C-30 and *Gliocladium* TUB F-498 in solid substrate fermentation. FPA = filter paper activity; BG = beta glucosidase activity in IU/g DW.



produced more enzymes than Rut C-30. TUB F-498 grew much faster and produced enzymes faster than Rut C-30 (Figure 2). Although in a 10 days fermentation Rut C-30 produced more cellulase and xylanase (but not beta glucosidase) than TUB F-498, the advantage of a short fermentation overrides the gain in enzyme production. Many current SSF processes suffer from long (10-20 days) fermentation time (9).

The comparison of enzyme production on wheat straw and sweet sorghum silage (Table 6) underlines the importance of substrate specificity for selecting a fungus for enzyme production. TUB F-498 was a much better enzyme producer on sweet sorghum, TUB F-486 performed better on wheat straw.

The excellent lignocellulolytic enzyme productivity of strain TUB F-498 in SSF was tested as a possible way for replacing commercial enzymes with in situ produced enzymes in the bioprocessing of sweet sorghum by enzyme assisted ensiling (ENLAC) (10). The process is illustrated in Figure 3, and described in detail elsewhere (1). The in situ enzyme was produced by SSF in aluminum trays on extracted sweet sorghum silage as described above. The enzyme activities of the 6 day fermentum were: FPA = 4.3; EG = 1450, BG = 13.4 and Xyl = 2370 IU/g DW. Two percent of the fermented substrate, as enzyme source, was mixed with freshly harvested sorghum and ensiled. The ENLAC process resulted in the conservation of the sugar content (see Table 7) and resulted in a 25% increase in sugar recovery by countercurrent extraction. The in situ enzyme gave as good results as the commercial NOVO enzymes Celluclast and Viscozyme.

Table 6. Lignocellulolytic enzyme production by solid substrate fermentation on different substrates.

	Wheat Straw					Extracted Sweet Sorghum Silage				
Strain	pH	FPA	EG	BG	Xyl	pH	FPA	EG	BG	Xyl
Rut C-30	6.0	10.7	2600	5.0	2200	5.4	6.8	1800	5.5	1100
TUB F-498	7.0	1.0	135	5.8	630	5.9	4.5	810	9.4	1520
TUB F-105	6.3	6.4	960	14.6	1640	6.0	4.0	710	5.2	1480
TUB F-482	6.5	3.8	1150	14.5	1160	5.7	3.6	600	7.8	1320
TUB F-486	6.2	4.6	840	10.4	1670	5.6	3.9	570	3.8	1030
Penicillium NRRL 2129	6.1	6.8	1510	31.8	2350	5.5	3.4	710	12.2	1580

Conditions: 10 days fermentation at 30°C, packing density 70g/250 ml for wheat straw, 80g/250 ml for sorghum in perforated plastic cups; inoculum 10^7 spores/g substrate; moisture content 75%. FPA = filter paper activity; EG = endoglucanase; BG = beta glucosidase; Xyl = xylanase; all activities in IU/gDW.

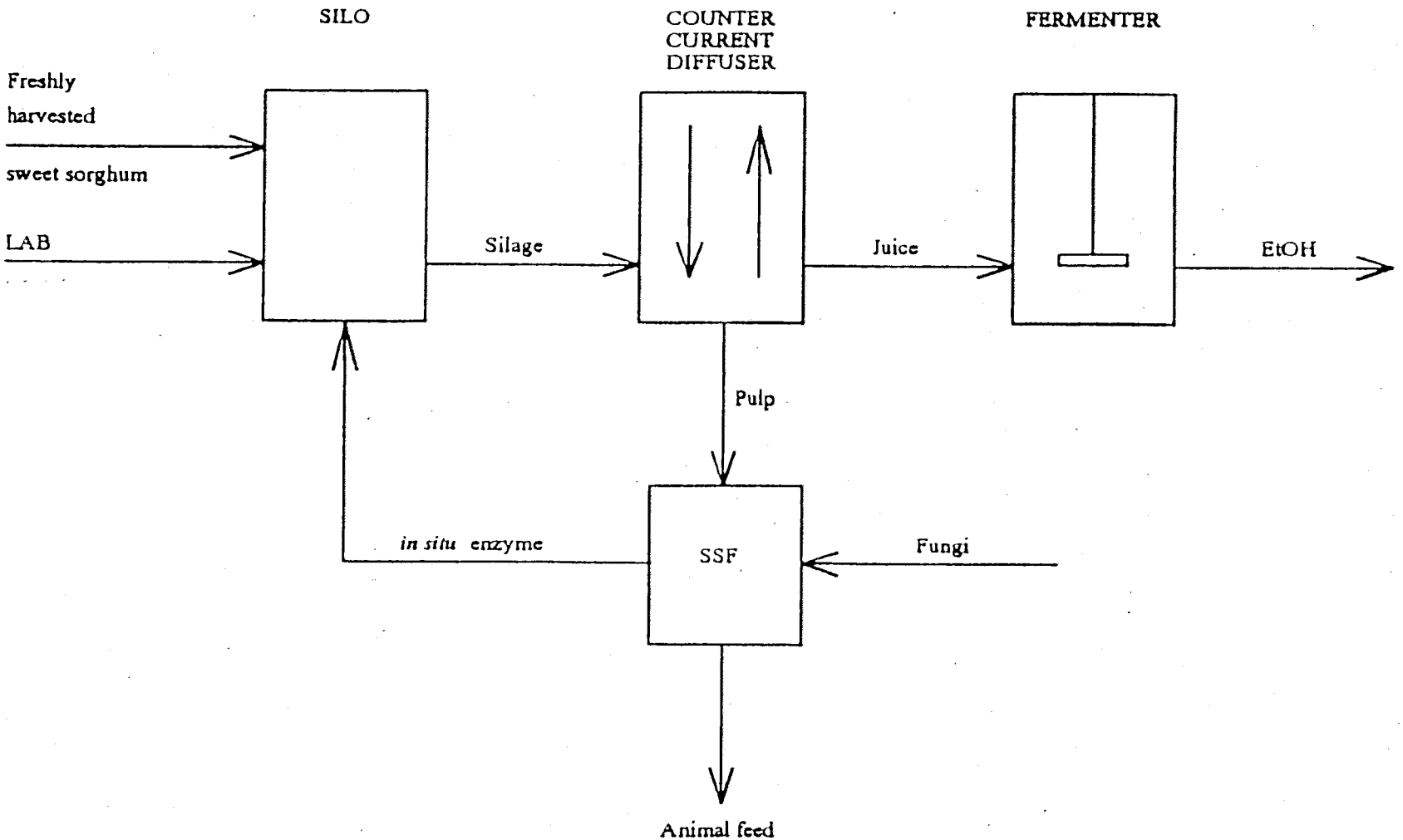


Figure 3. Integrated bioprocessing of sweet sorghum for ethanol production. LAB = lactic acid bacteria, SSF = solid substrate fermentation.

Table 7. Conservation of sugar content in enzyme assisted ensiling

Treatment	Reducing sugar g/kg DW 15 days	60 days	Dry weight (DW,g/kg)
Untreated sweet sorghum	201.1	25.0	212.7
Control silage	235.9	47.5	253.3
Silage with LAB	232.2	106.7	265.7
Silage + Cellulcast + Viscozyme + LAB	336.6	280.6	237.0
Silage + In situ enzyme + LAB	350.1	290.5	230.0

Reducing sugar content of freshly harvested sweet sorghum: 364 g/kgDW, DW content 314 g/kg
LAB: Lactic acid bacteria added at 10^5 CFU/g sorghum
Celluclast 1.5L NOVO: added at the level of 0.025%
Viscozyme®, NOVO: added at the level of 0.025%
In situ enzyme: pulp fermented with Gliocladium TUB F-498, added at the level of 2.0% (w/w)

The economic advantage of using in situ enzymes by SSF is illustrated in Tables 8-9. Table 8 compares the estimated cost of the in situ produced cellulase in sweet sorghum bioprocessing with the quoted bulk price of NOVO Celluclast 1.5 L. The in situ cellulase is about 30 times cheaper than Celluclast. In the illustrated ENLAC process, the total in situ enzyme cost is about $0.1 per metric ton substrate, compared with about $10 if commercial enzymes are used. This is shown in Table 9. If on site enzyme production would be used, e.g. in NREL's simultaneous saccharification and fermentation process, the cost of substrate, pretreatment and sterilization would have to be added, but the saving still would be significant, especially because of the high enzyme levels involved (c.f. letter to Dr. Steve Thomas 5.15.95). The in situ or on site enzyme is probably a better source for plant cell wall degradation than the commercial enzymes, due to the mentioned substrate specificity of the selected fungus.

Table 8. Comparison of commercial and in situ enzyme costs

1. Commercial cellulase, NOVO Celluclast 1.5L:

   Bulk price: $12.00/ℓ ; Activity: 80 IU/ml

   Unit price: $12.00 \frac{\$}{\ell} \cdot \frac{\ell}{1000 ml} \cdot \frac{1 ml}{80 IU} = 1.5 \times 10^{-4} \frac{\$}{IU}$

2. In situ enzyme by TUB F-498:

   Production cost: ~$6.00/MT (estimated from the production cost of compost)

   Activity: 4.5 IU cellulase/g DW fermentum

   Enzyme content of fermentum at 30% DW:

   Cellulase: $1.35.10^6$ IU/MT

   Cost of cellulase: $\frac{6.00}{1.35.10^6} \cdot \frac{\$}{MT} \cdot \frac{MT}{IU} = 4.4.10^{-6}\$/IU$

Table 9. Enzyme costs in enzyme assisted ensiling

Recommended level of enzymes used:

0.025% Celluclast 1.5L ($12/ℓ, 20,000 IU/MT)

0.025% Viscozyme 120L ($26/ℓ)

Commercial enzyme cost in process:

Celluclast: $250 \frac{ml}{MT} \times \frac{12 \$}{1000 ml} = \$3.0/MT$

Viscozyme: $250 \frac{ml}{MT} \times \frac{26 \$}{1000 ml} = \$6.5/MT$

Total enzyme cost: $9.5/MT

In situ enzyme cost in process:

A 2.0% addition of the in situ enzyme source to the substrate would provide 27,000 IU/MT cellulase and $2.7x10^6$ IU/MT xylanase, more than provided by the commercial enzymes at the 0.025% level.

Since the cost of the in situ enzyme is ~$6.00/MT, the total enzyme cost in the process would be:

$6.00 \frac{\$}{MT} \times 0.02 = \$0.12/MT$

CONCLUSIONS AND PROJECTIONS

The lignocellulolytic fungi isolated from this screening program have a high potential for cellulase and beta glucosidase production on natural substrates, such as pretreated poplar wood. The ratio of cellulase/beta glucosidase production by some of these fungi is more favorable for lignocellulose degradation than that of *T. reesei* Rut-C30. These fungi also have the potential for high level enzyme production by solid substrate fermentation, as an economical source of enzymes for some bioprocesses.

The full value of this study cannot be realized without further exploring the potential of the newly isolated strains. This requires genetic manipulation and a detailed study of the substrate specificity and physiology of these fungi under submerged and solid phase culturing conditions. It appears that these fungi behave very differently in submerged vs. solid phase culturing. The solid substrate fermentation appears to be a promising way to reduce currently exorbitant enzyme costs in bioprocessing. Solid substrate fermentation is eminently suitable for in situ or on site operations, therefore it deserves serious consideration. The necessary technology is available now, e.g. traditional malting in breweries and composting in automated facilities are well proven and economical technologies in the USA. The necessary fungi, as demonstrated in this study, could be exploited, and their enzyme productivity could be significantly boosted by genetic manipulations.

REFERENCES

1. Tengerdy, R.P., Szakacs, G. and Sipocz, J. (1995) Bioprocessing of sweet sorghum with in situ produced enzymes. Applied Biochem. and Biotechnol. (submitted)
2. Hach Inc. Manual (1990) Digesdahl Procedure. In: "Procedures Manual: Systems for Food, Feed and Beverage Analysis" pp. 15-21, Hach Inc., Loveland, CO.
3. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gardner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) A new protein assay using bicinchoninic acid. Anal. Biochem. 150:76-85.
4. Ghose, T.K. (1987) Measurement of cellulase activities. Pure and Appl. Chem. 59:257-268.
5. Kubicek, C.P. (1982) Beta glucosidase excretion by *Trichoderma pseudokoningii*. Arch. Microbiol. 132:349-354.
6. Bailey, M.J. and Nevalainen, K.M.H. (1981) Induction, isolation and testing of stable *T. reesei* mutants. Enzyme Microb. Technol. 3:153-157.
7. Bailey, M.J., Biely, P. and Poutanen, K. (1992) Interlaboratory testing of methods for assay of xylanase activity. J. Biotechnol. 23:257-270.
8. Montenecourt, B.S. and Eveleigh, D.E. (1977) Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. Appl. Environ. Microbiol. 34:777-782.
9. Chahal, D.S. (1991) Production of *Trichoderma reesei* cellulase system with high hydrolytic potential by solid state fermentation. In "Enzymes in Biomass Conversion", G.F. Leatham and M.E. Himmel, eds, Am. Chem. Soc., Washington, D.C., pp. 111-122.
10. Tengerdy, R.P., Linden, J.C., Wei Wu, Murphy, V.G., Baintner, F. and Szakacs, G. (1992) Plant processing by simultaneous lactic acid fermentation and enzyme hydrolysis. Appl. Biochem. Biotechnol. 34/35:309-316.